United States Department of Agriculture
Animal and Plant Health Inspection Service
Plant Protection and Quarantine

Guidelines for Isolation by Culture and Morphological Identification of *Phytophthora ramorum*

Adapted from SOP 2/27/2003 of Nancy Osterbauer, OR Dept. Agriculture
April 21, 2004

1. Laboratory Protocol

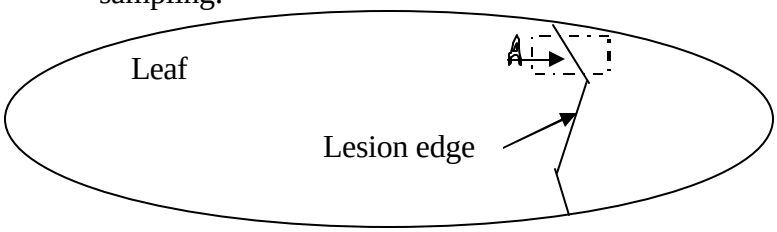
1.1 Log in samples by assigning each site a single identifying number or code (identifier). Record the State, identifier, the grower's license number (if applicable), the host(s), the inspector's initials as well as the location and date of inspection. It is suggested that samples be accompanied by a supplementary data sheet indicating the number of hosts present at each site. Save this data sheet in accordance with the NPDN format.

1.2 Identify the samples most symptomatic for *P. ramorum* infection for each host. ELISA will be utilized to pre-screen potentially symptomatic material for *Phytophthora* spp (see Appendix 5). Rinse the leaves in H_2O and then cut as described in Figure 1. If symptoms are not seen, or if an obvious lesion margin is not apparent, take samples from leaf tip, base or petiole. Change the H_2O between each site or between each subsample (host species or location) from the site. The rinse water must be sanitized or sterilized before disposal by autoclaving (see Section 3.2).

1.3 Label the underside of a PARP plate (Appendix 1) (near the edge) with the site's identifier and the date so sample identity is maintained.

1.4 Under a laminar flow hood, place one leaf section ("A" from Figure 1) onto the PARP plate. Slide the leaf section completely into the PARP so that the leaf piece is placed into the medium near the bottom of the plate. This technique forces the pathogen to grow through the medium near the plate bottom, which allows for easy observation of growth using a compound microscope (100X) to view the underside of the plate. Place a maximum of 10 leaf sections into the medium of each plate. Do NOT place leaf sections from different nurseries or from different subsamples (hosts or locations) within a site on the same plate. Seal the plate with parafilm and incubate in the dark at room temperature (68-70°F) in a secure location. Examine for *Phytophthora* growth after 4-7 days.

Figure 1. Dashed lines indicate cuts. Place piece A into PARP medium. Label the PARP plate appropriately. Place the remainder of the leaf back into the original sample bag and place in a cold room for storage when finished sampling.



1.5 Identifying *Phytophthora* to species:

1.5.1. Examine the leaf tissues plated onto PARP plates with a compound microscope or a high-powered stereo (dissecting) scope. Turn the plate upside down and look for the large chlamydospores. Use the 10x magnifying lens on a compound scope or the highest magnification on the stereoscope. A description of the fungus *in vitro* is given below in Appendix 2. *Phytophthora ramorum* will not form sporangia on PARP. Record your findings on the data sheet and in the associated database. Also be aware that *Pythium* species and *Mortierella* species will also grow on PARP, but differ in culture morphology (*Pythium* spp. are very fast growing with cytoplasmic streaming often visible and *Mortierella* spp. tend to form wavy hyphae).

1.5.2. Transfer any *P. ramorum*-like isolates to a V8 agar plate (Appendix 1). Place one isolate per plate. Label the plate with the appropriate sample number. Seal the plate with parafilm and incubate at room temperature (68-70°F). Save the original PARP plate.

1.5.3. Allow the isolate to grow on V8 for 4 days. Cut out three 6-mm plugs of the isolate and place in a sterile petri dish, mycelium side up. Add enough sterile tap H_2O to the petri dish to be level with the top of the plugs but not covering the mycelium. Carefully wrap petri dishes with parafilm.

1.5.4. Place the plugs + H_2O in the dark at room temperature (68-70°F) or an incubator set at = 18°C (64.5°F) and examine for sporangia after 48 hr. Sporangia will form on the edge(s) of the plug(s). Sporangia of *P. ramorum* are deciduous and can often be seen in the water.

1.5.5. Determine whether the organism is *Phytophthora ramorum* using chlamydospore and sporangial characteristics (see Appendix 2).

1.5.6. Record findings. Isolates identified as *Phytophthora ramorum* must be confirmed by an official identifier. At present, the APHIS labs (NIS and CPHST) in Beltsville are the only official identifying sites (Appendix 3).

2. Quality Control Criteria

2.1 Any positive identification of *Phytophthora ramorum* must be officially confirmed by two methods, morphology in addition to a PCR-based test, at the above-mentioned labs. Suspect positive V-8 cultures should be forwarded as indicated in Appendix 3.

3. Safety and Disposal:

3.1 Some of the components of PARP medium are **carcinogenic and extremely hazardous.** Laboratory personnel must follow OSHA's safety guidelines.

3.2 All plant samples, sample packaging material, and processed culture plates must be autoclaved at 121° C and 15 psi for 30 minutes prior to disposal.

3.3 All tools and other equipment must be sanitized and/or sterilized between samples.

Appendix 1: Medium

***Phytophthora*- selective medium (PARP):** Add 17g corn meal agar in 1000ml H_2O. Autoclave for 30 minutes (121° C/15 psi) then cool to 55°C. Once 55°C is reached add the following ingredients: 0.25g Ampicillin, 5ml PCNB solution, 1.0ml Rifampicin solution, 0.4ml Pimaricin (2.5% aqueous solution, Sigma Chemical cat. # P9703, P0440, or 80482) (Note: Some of these ingredients are carcinogenic and hazardous. Use caution when making this medium.)

The antibiotics in PARP are light sensitive. Plates should be poured under low-light conditions and stored in the dark. PARP medium can be stored for approximately 1 month.

Penta-Chloro-Nitro-Benzene (PCNB) stock solution: Carefully dissolve 2 g PCNB crystals in 400 ml 95% ethanol heated in a 70°C water bath.

Rifampicin stock solution: 0.01 g rifampicin dissolved in 1.0 ml DMSO.

Note: We've received numerous questions about the possibility of using a different formulation of PARP than was provided in the SOD Protocol for isolation of *Phytophthora ramorum*. Making small variations such as using methanol in place of DMSO are probably fine. However, we strongly prefer that you use the formulation in the protocol for consistency across all labs. If your lab has routinely used a variation of PARP for isolating *Phytophthora*, and you have documentation that shows that it is just as effective as our formulation of PARP, please retain that documentation in your files in the event it is needed for future reference.

V-8 Juice agar medium: Add 2 g of $CaCO_3$ to 30 ml V8 and stir for 5-min. Centrifuge for 20-min at 4,000 rpm or allow to settle overnight. Save supernatant and discard solid waste. Dilute to 1 L with dH_2O. Add 15 g of granulated agar and autoclave for 20-min at 121°C. Cool and pour.

Appendix 2: Description of *Phytophthora ramorum in vitro* on V8 Plates

Colonies on V-8 plates in dark 24-30 mm in approximately 6 days, aerial mycelium whitish, appressed, with concentric rings (Fig. 2)

Chlamydospores (Fig. 3) numerous, thin-walled, globose, hyaline to pale brown, mostly 46-60 um, terminal or intercalary.
Sporangia (Fig. 4) ellipsoid, elongate-ovoid, caducous, often with a short pedicel, semipapillate, hyaline, 45-65 x 21-28 µm, single or in clusters.

Mortierella species are more like *Mucor* species, (Figure 5), but are also isolated on PARP. *Pythium* species could also be isolated on PARP, and will grow very quickly to cover a plate in 3-5 days. Cytoplasmic streaming is often observed in *Pythium* hyphae. These should not be transferred to V8 plates.

Appendix 3: Submission and Identification of Suspect Positive Samples

Laboratory personnel should send suspect positive V-8 cultures to Dr. Palm, USDA/APHIS/PPQ/National Identification Service at the address below. For transport, all culture plates should be wrapped with Parafilm. Pack the cultures in a self-sealing plastic bag and place that bag(s) within a second plastic bag. Place in a sturdy cardboard box or similar container so that it does not get damaged during shipping and handling. Samples must be accompanied by a PPQ Form 391 (see below) or equivalent information. Samples should also include copies of any datasheets. Send samples by overnight express shipping (Fed Ex, UPS) and label the package as "URGENT and FRAGILE." If you have any questions concerning the procedure, please email the labs before shipping.

Dr. Mary E. Palm, PPQ National Mycologist (Mary.Palm@aphis.usda.gov)
USDA APHIS PPQ NIS
Room 329, Building 011A, BARC-West
10300 Baltimore Blvd.
Beltsville, MD 20705-2350

301-504-5327 PHONE
301-504-5810 FAX

Alternate NIS contact John McKemy (John.McKemy@aphis.usda.gov)
(301) 504-5280.

Morphological identification will be carried out by Drs. Palm or McKemy. The molecular testing will be done in the USDA-APHIS-PPQ-CPHST, National Plant Germplasm and Biotechnology Laboratory, Dr. Laurene Levy, Director, or at laboratories designated by APHIS-PPQ. Drs. Levy and Palm and their staffs will conduct the confirmation process as quickly as possible, generally within 3 days. Please do not call either of these staffs to inquire about the status of samples. The laboratory staffs will concentrate on the processing the samples.

Dr. Palm will notify the PPQ National Program Manager, who will ensure that findings are communicated to the promptly to the appropriate Regional and Headquarters PPQ staffs and State contacts. When the identity is confirmed, the sample is considered to be POSITIVE.

Specimen Submission Form (PPQ Form 391) that should accompany specimens submitted to the USDA/APHIS National Identification Laboratories can be found in Appendix 4.

Appendix 4: PPQ Form 391.

PPQ Form 391. Specimen Submission Form (PPQ Form 391) to accompany specimens submitted to the USDA/APHIS National Identification Laboratories.

This report is authorized by law (7 U.S.C. 147a). While you are not required to respond your cooperation is needed to make and accurate record of plant pest conditions.

FORM APPROVED
OMB NO. 0579-0010

U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE

SPECIMENS FOR DETERMINATION

Instructions: Type or print information requested. Press hard and print legibly when handwritten. Item 1 assign number for each collection beginning with the year, followed by collector's initials and collector's number. Example (collector, John J. Dingle); 83-JJD-001. Pest Data Section – Complete Items 14, 15 and 16 or 19 or 20 and 21 as applicable. Complete Items 17 and 18 if a trap was used.

FOR IIBIII USE
Lot No.

PRIORITY

1. COLLECTION NUMBER

2. DATE — MO | DA | YR

3. SUBMITTING AGENCY

☐ State ☐ PPQ ☐ Other _____

SENDER AND ORIGIN

4. NAME OF SENDER

6. ADDRESS OF SENDER

CITY, STATE ZIP

INTERCEPTION SITE

5. TYPE OF PROPERTY *(Farm, Feedmill, Nursery, etc.)*

7. NAME AND ADDRESS OF PROPERTY OR OWNER

COUNTRY/COUNTY

PURPOSE

8. REASON FOR IDENTIFICATION *("x" ALL Applicable Items)*

A. ☐ Biological Control (Target Pest Name)	E. ☐ Livestock, Domestic Animal Pest
B. ☐ Damaging Crops/Plants	F. ☐ Possible Immigrant (Explain in Remarks)
C. ☐ Suspected Pest of Regulatory Concern (Explain in Remarks)	G. ☐ Survey (Explain in Remarks)
D. ☐ Stored Product Pest	H. ☐ Other (Explain in Remarks)

9. IF PROMPT OR URGENT IDENTIFICATION IS REQUESTED, PLEASE PROVIDE A BRIEF EXPLANATION UNDER "REMARKS".

HOST DATA

10. HOST INFORMATION

NAME OF HOST *(Scientific name when possible)*

11. QUANTITY OF HOST

NUMBER OF ACRES/PLANTS

PLANTS AFFECTED *(Insert figure& indicate number or percent)* ☐ Number ☐ Percent

12. PLANT DISTRIBUTION

☐ LIMITED
☐ SCATTERED
☐ WIDESPREAD

13. PLANT PARTS AFFECTED

☐ Leaves, Upper Surface	☐ Trunk/Bark	☐ Bulbs, Tubers, Corns	☐ Seeds
☐ Leaves, Lower Surface	☐ Branches	☐ Buds	
☐ Petiole	☐ Growing Tips	☐ Flowers	
☐ Stem	☐ Roots	☐ Fruits or Nuts	

PEST DATA

14. PEST DISTRIBUTION

☐ FEW
☐ COMMON
☐ ABUNDANT
☐ EXTREME

15. ☐ INSECTS ☐ NEMATODES ☐ MOLLUSKS

NUMBER SUBMITTED	LARVAE	PUPAE	ADULTS	CAST SKINS	EGGS	NYMPHS	JUVS.	CYSTS
ALIVE								
DEAD								

16. SAMPLING METHOD

17. TYPE OF TRAP AND LURE

18. TRAP NUMBER

19. PLANT PATHOLOGY – PLANT SYMPTOMS *("X" one and describe symptoms)*

☐ ISOLATED ☐ GENERAL

20. WEED DENSITY

☐ FEW ☐ SPOTTY ☐ GENERAL

21. WEED GROWTH STAGE

☐ SEEDLING ☐ VEGETATIVE ☐ FLOWERING/FRUITING ☐ MATURE

22. REMARKS

23. TENTATIVE DETERMINATION

24. DETERMINATION AND NOTES *(Not for Field Use)*

FOR IIBII USE
DATE RECEIVED
NO.
LABEL
SORTED
PREPARED
DATE ACCEPTED
RR

SIGNATURE DATE

Fig. 2. Fifteen day old culture of *P. ramorum* on V-8.

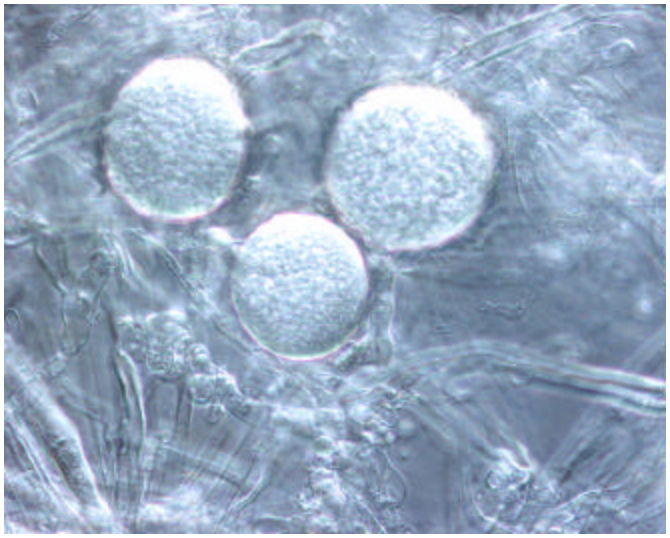


Fig. 3. Chlamydospores of *P. ramorum* (DIC optics).

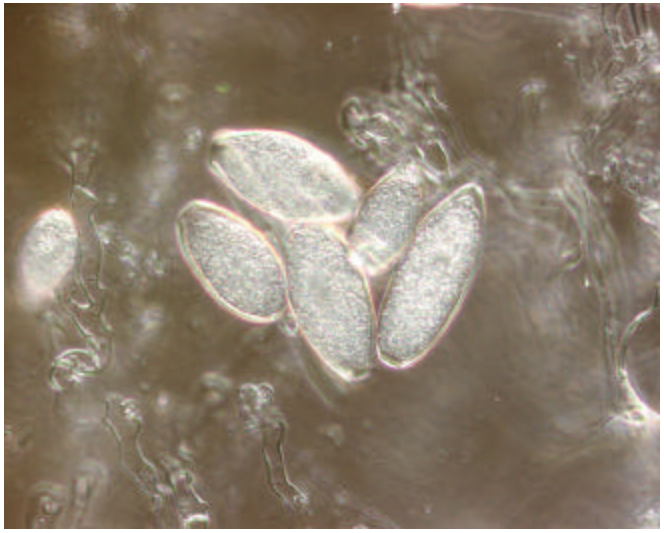


Fig. 4. Semi-papillate sporangia of *P. ramorum* (DIC optics)

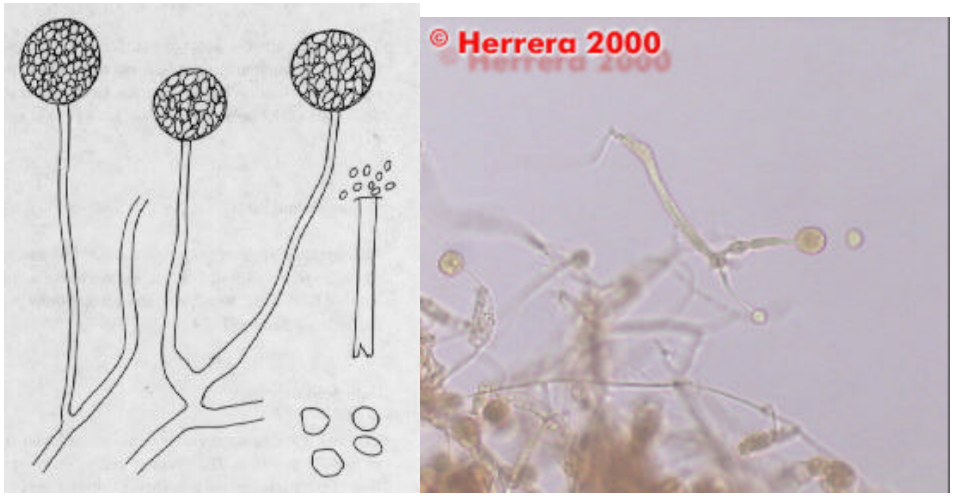


Fig. 5. Examples of *Mortierella* species
(http://www.botany.utoronto.ca/researchlabs/ mallochlab/malloch/Moulds/Mortierella.html and http://www2.truman.edu/~jherrera /Zygomycetes/Mortierella/Mortierella.html)